Review

CD4⁺Foxp3⁺ regulatory T cell therapy in transplantation

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Regulatory T cells (Tregs) are long-lived cells that suppress immune responses in vivo in a dominant and antigen-specific manner. Therefore, therapeutic application of Tregs to control unwanted immune responses is an active area of investigation. Tregs can confer long-term protection against auto-inflammatory diseases in mouse models. They have also been shown to be effective in suppressing alloimmunity in models of graft-versus-host disease and organ transplantation. Building on extensive research in Treg biology and preclinical testing of therapeutic efficacy over the past decade, we are now at the point of evaluating the safety and efficacy of Treg therapy in humans. This review focuses on developing therapy for transplantation using CD4⁺Foxp3⁺ Tregs, with an emphasis on the studies that have informed clinical approaches that aim to maximize the benefits while overcoming the challenges and risks of Treg cell therapy.

Keywords: regulatory T cells, transplantation, cell therapy, immune tolerance

Introduction

The immune system is a formidable barrier to the success of cell and organ transplantation. Immunosuppressive medications are necessary to protect transplanted cells and organs from graft rejection. Although immunosuppressive regimens continue to be refined with significant reduction in the incidence of acute rejection, improvement in long-term outcomes has stagnated, in part, due to the morbidity and mortality caused by non-specific immunosuppression (Feng, 2008). The traditional approach to immunosuppression has emphasized the control of effector T cell responses. The relatively recent elucidation of regulatory T cells (Tregs) and their importance in suppressing autoimmunity and alloimmunity has inspired new thinking in managing alloresponses. Emerging data suggest that designing immunosuppression regimens with a ‘Treg-centric’ approach to promote regulation may favor induction of graft tolerance and improve long-term graft outcomes (Wood and Sakaguchi, 2003; Bluestone and Tang, 2004; Walsh et al., 2004; Kang et al., 2007; Sagoo et al., 2008; Waldmann et al., 2008; Long and Wood, 2009).

Unlike generalized immunosuppressive regimens, Tregs are long-lived and function in a dominant and antigen-specific manner. Thus, therapeutic infusion of Tregs has the potential to induce long-term donor-specific tolerance without impeding desired immune responses to pathogens and tumors in transplant patients. Research in animal models has demonstrated that Tregs can be used to treat many auto-inflammatory diseases such as type 1 diabetes, inflammatory bowel disease, systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, and autoimmune gastritis. In addition, Treg therapies have been found to be efficacious in controlling alloimmune responses in the settings of graft-versus-host disease (GvHD), as well as organ and cell transplantation in animal models.

A key advance for Treg therapy in humans is the finding that Tregs can be isolated and expanded in vitro while maintaining immunoregulatory function (detailed below). As of mid-2011, three clinical trials evaluating the safety and efficacy of Tregs in treating GvHD have been reported, all demonstrating promising safety and potentially efficacy profiles (Trzonkowski et al., 2009; Brunstein et al., 2010; Di Ianni et al., 2011). There is great interest by multiple investigators to consider wider application of Treg therapy in other disease settings such as autoimmunity and solid organ transplantation (Leslie, 2011). At this juncture, it is helpful to review on the history of Treg therapy in preclinical models and strategies for application in humans. In this review, we will focus on Treg therapy in the setting of tissue transplantation, with an emphasis on potential risks and benefits as well as parameters with direct relevance to the design of clinical Treg therapies.

The intertwined fields of transplantation and immune regulation

Immune tolerance was first conceptualized based on studies of immune responses to allogeneic (and xenogeneic) antigens. The landmark observation by Ray Owens in 1945 that dizygotic
(therefore allogeneic) twin calves were tolerant to each other’s blood cells was reinforced by experimental evidence of tolerance to skin allografts in mice, chickens, and cattle in the early 1950s. Furthermore, ‘suppressor T cells’, analogous to current day Tregs, and capable of transferring dominant tolerance were first demonstrated in experimental models of transplantation by Gershon and Kondo in the early 1970s. Despite much skepticism within the immunology community regarding the very existence of ‘suppressor’ T cells in the 1980s and 1990s, investigations on the cellular basis of transplantation tolerance continued based on the reproducible finding that T cells from tolerant mouse could transfer dominant and infectious tolerance to a new host. Dominant suppression refers to the ability of the suppressor cells to suppress non-tolerant T cells. Infectious tolerance denotes the phenomenon that the tolerance can spread to T cells with a new distinct specificity when the new antigen is present within the same graft. It is noteworthy that while the suppressor T cell literature in the early 1980s dominated by the CD8+ suppressor T cells (Dorf and Benacerraf, 1984), many transplant researchers found that CD4+ T cells were responsible for the donor-specific dominant and transferable tolerance and Hall et al. (1990) arrived at the conclusion that transplant tolerance was mediated by CD4+CD25+ cells, 5 years prior to the demonstration that CD4+CD25+ Tregs were responsible for self-tolerance (Sakaguchi et al., 1995). The discovery of Foxp3 as the transcriptional driver of Treg function propelled the study of Tregs from a boutique field within auto- and alloimmunity to the forefront of immunological research (Sakaguchi et al., 1995; Fontenot et al., 2003; Hori et al., 2003; Khattrir et al., 2003). By the early 2000s, the fields of self-tolerance and transplantation tolerance finally converged (Waldmann and Cobbold, 2001; Wood and Sakaguchi, 2003). It became clear that the CD4+CD25+ cells so vital to self-tolerance were also the mediators of transferrable tolerance to allogeneic transplants.

Since its inception in the mid-1940s to the clinical translation in the late-2000s, the field of immune regulation has been closely connected to transplantation. Transplantation offers convenient models to study immune regulation, especially in the era before the cloning of T cell receptors and the availability of transgenic mouse models. The advances in the field of immune regulation in the past 20 years, in turn, may offer the field of clinical transplantation new therapeutic options to control rejection and induce tolerance to transplanted antigens.

**Treg control of transplant rejection: preclinical experience**

When considering moving Treg therapy to the clinic to induce transplant-specific tolerance, it is helpful to review experiences in preclinical models. Issues such as the types and specificity of the Tregs, timing and location of Treg actions, stability and plasticity of Tregs, and adjunct therapies that synergize with therapeutic Tregs are important to the optimization of clinical trial designs using Tregs. Many types of Tregs have been identified beyond the CD4+CD25+Foxp3+ Tregs and some have demonstrated efficacy in models of transplantation. Our review will be limited to the CD4+Foxp3+ Treg subset.

**Treg alloantigen specificity**

There are two types of alloreactive CD4+ T cells, including Tregs, depending on the MHC class II restriction. The direct alloreactive CD4+ T cells are unique to transplantation in that their T cell receptors ‘directly’ bind to donor (i.e. foreign) MHC class II expressed on donor cells. The indirect alloreactive CD4+ T cells are like other T cells and recognize allogeneic peptides presented by the recipient’s MHC class II molecules on self-antigen presenting cells. The frequencies of direct alloreactive T cells are present at orders of magnitude higher than the frequencies of cells with indirect specificity, and this is likely to be the case for alloreactive Tregs. The presence of alloreactive Tregs is evident from the observation that Tregs isolated from naive mice can suppress responses to alloantigen in vitro and protect against allograft rejection when transferred in vivo (Davies et al., 1999; Taylor et al., 2001; Earle et al., 2005). Depletion of Tregs using anti-CD25 antibody in naive hosts prior to transplantation appears to hasten graft rejection (Cohen et al., 2002; Benghiat et al., 2005; Bolton, 2005). A recent study reported the frequency of direct alloreactive Tregs to be over 10% (Lin et al., 2008). Despite the high prevalence of the direct alloreactive Tregs, most allografts are rejected acutely demonstrating that Tregs present in the naive recipients are not sufficient to prevent rejection. Various transplant tolerance-inducing protocols promote the expansion and/or conversion of Tregs, further increase the breadth of the Treg repertoire and dominance of Treg control over the effector-mediated rejection. It is important to note that in many instances, tolerance-inducing protocols expand Tregs with the indirect specificity (Wise et al., 1998; Ochando et al., 2006; Verginis et al., 2008), suggesting that long-term allograft tolerance is primarily mediated through the indirect pathway (Hara et al., 2001; Yamada et al., 2001; Callaghan et al., 2007; Gokmen et al., 2008).

Since transplant tolerance is associated with donor-specific Treg expansion, it is conceivable that administration of donor-specific Tregs may prolong graft survival and/or induce tolerance. Indeed, this has been demonstrated in a variety of experimental transplant models (Trenado et al., 2003; Golshayan et al., 2007; Joffre et al., 2008; Tsang et al., 2008; Nadig et al., 2010; Brennan et al., 2011; Saggoo et al., 2011). In most of these studies, donor antigen-specific Tregs are more effective than polyclonal Tregs, and most used Tregs of direct specificity. Two independent studies found that a combination therapy using Tregs of direct and indirect specificities was superior to that relied on Tregs of direct specificity only (Joffre et al., 2008; Tsang et al., 2008). Given the importance of the indirect pathway in mediating tolerance induced by various protocols, it is not surprising that additional indirect specificity is advantageous. However, there is a paucity of data on Tregs with only indirect specificity and ample experimental support for the efficacy of Tregs with only direct specificity. Part of this disparity may be due to the relative ease in selectively expanding direct Tregs than indirect Tregs. It is possible that the adoptively transferred direct Tregs effectively controlled the acute rejection mediated by the direct effector T cells, allowing indirect Tregs to emerge from endogenous source to mediate long-term protection. Taken together, preclinical experiments thus far demonstrate that both mouse and human Tregs with direct specificity can be selectively
expanded from a polyclonal pool and these specific Tregs are more effective than polyclonal Tregs at preventing allograft rejection and inducing tolerance.

**Timing of Treg function**

Most, if not all, preclinical Treg therapy studies published thus far administered Tregs prior to or at the time of transplant, implying a lack of efficacy when Treg introduction is delayed until after graft implant. This is in sharp contrast to Treg control of autoimmune disease and inflammatory bowel disease where Treg therapy after disease initiation can effectively reverse the disease course (Tang and Bluestone, 2006). The intensity of the anti-allograft response and the fragility of the transplanted grafts may both contribute to the lack of efficacy when Tregs are delayed. A recent study analyzing the dynamics of alloimmune response in vivo using an elegant imaging approach demonstrated rapid invasion of effector cells in the grafts followed by delayed arrival of Tregs that were ineffective at controlling tissue damage (Fan et al., 2010). In contrast, when the recipient mice were treated with anti-CD40L mAb and rapamycin, a protocol that induced long-term graft acceptance, effector T cell infiltration was delayed and reduced without affecting the kinetics of Treg migration to the graft. As a result, over 30% of the infiltrating T cells were Tregs. While most of the Tregs infiltrating the grafts were natural Tregs, the presence of recently converted adaptive Tregs increased at later time points, suggesting a spread of Treg repertoire under the tolerogenic condition. Such activation kinetics allows the immune system to mount a response against foreign antigens first and the delayed arrival of Tregs serves as a negative feedback loop to prevent excessive collateral damage to the surrounding tissue. In most transplant settings, the arrival of the endogenous Tregs at the graft sites is too late to restore graft function, resulting in irreversible graft loss.

In this regard, it is interesting to speculate that tolerance may be achieved relatively easier with certain graft types because of their ability to withstand immune attacks. For example, allogeneic liver grafts are spontaneously tolerated in mouse models despite vigorous activation of alloimmune responses. Histological analysis shows clear evidence of initial graft damage, which resolves after 2 weeks as the immune response wanes (our unpublished data). This is analogous to the results reported using liver expression of a model self-antigen where mice recover from the initial tissue attack with the emergence of antigen-specific Tregs leading to tolerance (Knoechel et al., 2005). Clinically, although liver grafts are not spontaneously accepted without immunosuppression, they are more tolerogenic than other graft types due to their resilience and regenerative ability.

The requirement for early administration of Tregs may make Treg therapy difficult to implement in the clinical setting, particularly if prior expansion of donor-specific Tregs is planned. Since donor cells are required to selectively expand donor-specific Tregs and at least several weeks are required for adequate expansion (Sagoo et al., 2011), donor-specific Treg therapy at the time of transplant is only possible for patient with living donors. In this regard, it is worth pointing out that most Treg therapy in mouse transplant models reported to date did not involve additional maintenance immunosuppressive drugs. Such a regimen is clearly not permissible in human patients before the safety and efficacy of Treg therapy in humans are established. It is not clear if the use of induction therapy and/or immunosuppression would allow delayed administration of Tregs without losing efficacy. Therefore, it is worthwhile to revisit the issue of timing of Treg infusion in preclinical models with various adjunct immunosuppressive treatments.

**Location of Treg function**

The site of Treg action has been extensively investigated in both the autoimmune and transplant settings. In the autoimmune setting, Tregs are initially activated in the draining lymph nodes (LNs) to prevent priming and clonal expansion of autoreactive effectors (Tang and Bluestone, 2006). Once tissue inflammation breaks out, Tregs also traffic to inflamed tissue and exert their suppressive activity peripherally. In the transplant setting, Treg LN homing and their ability to traffic to grafts are both required for their protection against graft rejection (Lin et al., 2002; Cobbold et al., 2004; Ochando et al., 2005). Interestingly, one report showed that, in a mouse islet transplant model, therapeutic Tregs function initially at the graft site, and then traffic to the draining LN via afferent lymphatics and continue to exert their suppressive function there (Zhang et al., 2009). The early infiltration of Tregs in the grafts prevents the exit of donor-derived dendritic cells to the draining LN, thereby reducing alloimmune priming. This apparently reversed sequence of actions of Tregs may be unique to the transplant setting when tissue inflammation occurs as a result of surgery without prior T cell activation. Inflammation induced by surgical trauma to the host and ischemia-reperfusion injury of the graft may directly recruit alloreactive T cells to the graft site prior to clonal expansion in the LN. Because of the high precursor frequency of alloreactive T cells, graft damage can occur in the absence of secondary lymphoid organs, although less efficiently (Lakkis et al., 2000; Zhou et al., 2003). It is conceivable that transplant rejection is a vicious cycle of graft damage, release of donor antigens, priming of alloreactive T cells in lymphoid organs, infiltration of effector T cells into the graft leading to further shedding of graft antigens, and expanded alloimmune activation, and further attack on the graft until the graft is destroyed. Therapeutic Tregs likely respond to the same inflammatory cues and directly traffic to the graft site after injection to intervene early before the vicious cycle of anti-graft response spins out of control.

**Stability and plasticity of Tregs**

Once thought to be a stable sub-lineage of CD4+ T cells, there is evidence now that Tregs may be plastic (Zhou et al., 2009a, b). This obviously poses concerns about the safety and efficacy of Treg therapy. The stability and function of Tregs critically depends on high expression of Foxp3, which is controlled at epigenetic and protein levels. Between the Foxp3 promoter and the first exon lies a stretch of highly conserved non-coding sequence that is differentially methylated in Tregs versus Tconv CD4+ cells (Baron et al., 2007; Floess et al., 2007). This sequence, referred to as Treg-specific demethylated region (TSDR) or conserved non-coding sequence 2, is crucial to maintaining high Foxp3 expression in Tregs (Zheng et al., 2010). The CpG-rich DNA in TSDR is demethylated in Tregs, allowing binding of transcription factor complex that contains CREB/ATF, STAT5, ETS-1, and Foxp3 itself to maintain transcriptional activity (Baron et al., 2007; Polansky
et al., 2010). Adaptive Tregs induced to express Foxp3 by in vitro exposure to TGF-β have methylated TSDR, and are not stable Tregs (Floess et al., 2007; Wieczorek et al., 2009). In addition to this epigenetic mechanism in controlling Foxp3 expression, Foxp3 function and expression is also controlled at the post-translational level by acetylation of its many lysine residues (Wang et al., 2009; Xiao et al., 2010). The acetylation of lysine residues in Foxp3 prevents their polyubiquination and subsequent degradation by proteosomes (van Loosdregt et al., 2011). Additionally, Foxp3 acetylation also promotes binding to DNA. Thus, stability of Foxp3 expression in Tregs is controlled at epigenetic and protein levels to ensure the stability of the lineage.

Despite this fortified system to stabilize Tregs, emerging evidence suggests that Tregs can lose Foxp3 expression and acquire the ability to make effector cytokines under some experimental conditions. Foxp3 prevents Treg expression of IL-17 by antagonizing the Th17-specific transcription factor RORγ. Exposure of Tregs to IL-6 and IL-1 in vitro lifts the Foxp3 repression of RORγ, leading to their expression of the IL-17 (Yang et al., 2008). In vivo, loss of Foxp3 has been found in the settings of autoimmune disease (Zhou et al., 2009c), fetal acute infections (Oldenhove et al., 2009), TLR stimulation (Sharma et al., 2010), IL-2 deficiency (Rubtsov et al., 2010), and homeostatic proliferation (Komatsu et al., 2009). Human Tregs stimulated repeatedly in vitro with anti-CD3 and anti-CD28 antibodies lose Foxp3 expression (Hoffmann et al., 2009). Thus, loss of Foxp3 and Treg instability can clearly happen in pathological conditions, and it is important to determine the origin of the unstable Tregs, extrinsic triggers of their instability, and the fate of these ‘exTregs’. A heterogeneity model has been recently proposed to explain the origin of ‘exTregs’ (Hori, 2011). This model suggests that Foxp3+ cells are heterogeneous and may contain cells that are not fully committed to the Treg lineage. Consistent with this idea, epigenetic analysis shows that thymic Tregs and adaptive Tregs induced in culture with TGF-β have partially methylated TSDR despite high levels of Foxp3 expression (Floess et al., 2007; Wieczorek et al., 2009), indicating a time lapse between Foxp3 expression and the commitment to the Treg lineage in the thymus and possibly in the periphery. Lack of further commitment cues and/or exposure to inflammatory cytokines and/or IL-2 deprivation at this developmental stage may cause these cells to abort Treg development program and lose suppressive function, and subsequently become anergic, take on an effector phenotype, or die. Such pruning mechanism may help to limit Treg repertoire to those antigens that are persistently tolerogenic. This may be especially important for human Foxp3+ cells because most human CD4+ T cells transiently up-regulate Foxp3 after activation (Walker et al., 2003; Tran et al., 2007; Broady et al., 2009), but TSDR locus is methylated in these activated Tconv cells (Baron et al., 2007; Komatsu et al., 2009; Mc Clymont et al., 2010). Quantitative assessment of TSDR may allow us to estimate the frequencies of these partially committed Tregs in various healthy and disease settings and would provide a useful tool for assessing the stability of Tregs manufactured for therapeutic use in humans.

It is important to make the distinction between Treg instability and functional specialization. Under inflammatory conditions, Tregs can acquire the ability to produce effector cytokines while maintaining high expression of Foxp3 and suppressive activity. Recent experimental evidence suggests that Treg expression of transcription factors and cytokines specific for Th1, Th2, and Th17 cells enables them to control inflammation mediated by Th1, Th2, and Th17 cells by responding to the same inflammatory cues (Campbell and Koch, 2011). For example, expression of T-bet and IFN4, transcription factors for Th1 and Th2 cells, are required for Tregs to efficiently control Th1- and Th2-mediated immune pathology, respectively (Koch et al., 2009; Zheng et al., 2009). Additionally, IL-17 is produced by a subset of highly suppressive human Tregs that express CCR6, a chemokine receptor preferentially used for recruiting Th17 cells to the site of inflammation (Voo et al., 2009). In the transplant setting, IFNγ production by Tregs was shown to be essential for their control of allograft rejection (Sawitzki et al., 2005). Thus, Treg expression of effector cytokines alone cannot be simply viewed as a marker of plasticity or lack of stability, but likely a hallmark of their functional specialization. A key distinction between specialized Tregs and plastic Tregs is the stable expression of Foxp3.

Adjunct immunosuppressive therapy

Preclinical data discussed thus far show that Treg therapy alone, antigen-specific or polyclonal, is not sufficient to protect a major MHC-mismatched graft from rejection in a normal, otherwise untreated host. Adjunct immunosuppressive therapy is needed to create a therapeutic window for Tregs to induce tolerance. Drugs that induce a significant reduction in donor-reactive Tconv CD4+ and CD8+ T cells, especially those with direct specificity, are likely essential prerequisites for Tregs to induce and maintain graft tolerance (Wells et al., 1999; Xia et al., 2008). Various approaches have been used to reduce alloreactive T cell clonal size. The most straightforward is the use of T cell depleting antibodies. Thymoglobulin is a rabbit polyclonal anti-T cell antibody preparation commonly used in transplant patients to treat acute rejections. It has been used with increasing frequency at the time of transplant as an induction therapy in high-risk patients or to enable delay or minimization of other immunosuppressive drugs (Deeks and Keating, 2009). Similarly, mAbs against CD3 or CD52 have also been used to treat acute rejections and as induction agents (Kirk, 2006). In fact, the mouse anti-human CD3 antibody OKT3 was the first mAb approved for therapy in humans in 1986. However, its use is limited due to severe side effects secondary to pan T cell activation after the initial dose and generation of neutralizing antibodies. Several engineered forms of humanized anti-CD3 antibodies with mutated Fc regions have been made that showed similar efficacy in deleting T cells and reversing graft rejections without the associated toxicity and neutralizing antibodies. In mice, the engineered FcR non-binding antibodies preferentially delete activated effector T cells while stabilizing Tregs (Penaranda et al., 2011). Similarly, thymoglobulin induction therapy is also reported to preferentially kill Tconv cells resulting in increased proportion of Tregs (Lopez et al., 2006; Morelon et al., 2010). Thus, thymoglobulin and anti-CD3 are compatible with Treg therapy as induction agents to reduce clonal size of donor-reactive T cells and may have the added benefit of preserving endogenous Tregs in the hosts.
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Non-specific T cell deletion leads to homeostatic proliferation of residual T cells and their concomitant acquisition of memory phenotype that resists tolerance induction in experimental models (Wu et al., 2004; Moxham et al., 2008). Whether this occurs in human transplant recipients is yet to be determined, because T cell depletion in transplant patients is always followed by maintenance immunosuppression, which may change how T cells behave in a lymphopenic environment. Analyses of peripheral blood memory and effector T cells after thymoglobulin treatment in patients showed variable results with some investigators reporting an increase in the proportion of memory and effector cells while others showed a reduction in effector and memory T cells (Sener et al., 2009; Gurkan et al., 2010; Morelon et al., 2010; our unpublished data). Differences in the choice of maintenance immunosuppression may influence the proportion of memory and effector T cells after T cell depletion (Morelon et al., 2010). A particularly encouraging finding is that Treg therapy and other experimental tolerogenic treatments have been shown to restore tolerance in lymphopenic hosts (Neujahr et al., 2006; D’Addio et al., 2010). Thus, the choice of adjunct immunosuppressive regimen after T cell depletion will likely have significant impact on tolerance induction and therapeutic efficacy of Tregs.

A strategy that can selectively inactivate donor-reactive T cells without massive non-specific T cell depletion would be ideal to combine with Treg therapy. Although there is no such proven strategy in the clinical setting, several approaches have been successful in experimental models. For example, using donor bone marrow transplantation to achieve high levels of donor chimerism effectively deletes donor-reactive T cells (Cosimi and Sachs, 2004; Sykes, 2009). Such an approach usually requires drastic myeloablative conditioning that includes radiation and anti-lymphocyte antibodies. Using bone marrow chimerism to induce tolerance has been tested in clinical trials with demonstrable tolerance in some, but not all, patients (Kawai et al., 2008; Scandling et al., 2008; LoCascio et al., 2010; Spitzer et al., 2011). Of note, transplant tolerance in patients with high level of chimerism is associated with deletion of donor-reactive T cells and later emergence of Foxp3 mRNA in the grafts. It has been suggested that rapid and efficient deletion of donor-reactive T cells in hosts with high level of chimerism may preclude the generation of Tregs (Kurtz et al., 2004), thus addition of Tregs in this setting may be synergistic.

Transfusion of donor whole blood, also known as donor-specific transfusion, has reported tolerogenic effects in animal models, especially when combined with costimulation blockade. Combining donor-specific transfusion with anti-CD40L has been shown to pre-emptively delete donor-reactive T cells of the direct and indirect pathway (Quezada et al., 2003; van Maurik et al., 2004). In addition, donor-specific transfusion combined with CD4 blockade or exposure to altered donor antigens prior to transplantation may render donor-reactive T cells unresponsive thus effectively reducing donor-reactivity (Chen et al., 2004; Luo et al., 2008). Therefore, as Treg therapy moves to the clinical arena, there are several opportunities to enhance efficacy by selectively depleting the highly frequent and potentially pathogenic alloreactive Tconv CD4+ and CD8+ cells.

In addition to induction therapy to reduce donor-reactive T cells, maintenance immunosuppression will likely be needed, at least for a short period after transplant, especially in early clinical trials where safety is a major concern. It is important to note that Treg function depends on activation through their TCR and costimulatory molecules. Additionally, therapeutic Tregs most likely function through infectious tolerance by inducing endogenous regulatory populations with a wider array of specificity and tolerogenic properties. Partial T cell activation may promote infectious spread of suppression (Padberg et al., 1987; Sho et al., 2005). Therefore over-immunosuppression will likely interfere with Treg function, negatively impact their stability, and even abrogate tolerance induction (Li et al., 1999). In addition, the choice of immunosuppressants will be critical, as they have varying effects on Tregs (Demirkiran et al., 2008; Fourtounas et al., 2010). The ideal adjunct immunosuppressive therapy would target effector T cells while minimally interfering with Treg homeostasis and function. Widely used calcineurin inhibitors, cyclosporin A and tacrolimus, block calcium-induced nuclear translocation of transcription factor NFAT. Although NFAT activation is essential for Tconv cell activation, it is also important for activation-induced cell death of Tconv cells, induction of Foxp3 expression, and Treg function (Rudensky et al., 2006; Tone et al., 2008). Calcineurin inhibition also suppresses IL-2 secretion, which is important for Treg homeostasis. Many reports show that chronic use of calcineurin inhibitors is associated with a decline of Tregs in patients, and conversion from calcineurin inhibitors to other immunosuppressive drugs leads to recovery of Tregs (Baan et al., 2005; Pascual et al., 2008; Demirkiran et al., 2009). It is noteworthy that some studies found that Tregs are more resistant to calcineurin inhibition than Tconv cells (Brandt et al., 2009; Calvo-Turrubiartes et al., 2009), suggesting that the effect of calcineurin inhibition on the balance of effector T cell and Treg function may be dose dependent and low dose may be permissive and even supportive for Treg function.

Rapamycin inhibits the mammalian target of rapamycin (mTOR), which is downstream of phosphatidylinositol 3-kinase (PI3K), a signaling molecule activated by CD28 or IL-2 receptor engagement in T cells (Thomson et al., 2009). IL-2 receptor engagement activates both PI3K–mTOR and Janus kinase–STAT pathways. Biochemical analysis of IL-2 signaling in Tregs shows that the PI3K–mTOR pathway is attenuated, whereas the Janus kinase–STAT pathway remains intact, suggesting that Tregs preferentially signal through the latter and may be resistant to mTOR inhibition (Zeiwer et al., 2008). This notion is supported by genetic ablation and cellular experiments that demonstrate mTOR deficiency or addition of rapamycin favors the outgrowth and function of Tregs (Battaglia et al., 2005; Delgoffe et al., 2009). Consistent with these in vitro observations, the use of rapamycin in mouse transplant models was found to promote Tregs (Coenen et al., 2007; Gao et al., 2007; Kopf et al., 2007). In transplant patients, the use of rapamycin-based immunosuppression is also associated with an increase in Tregs when compared with patients on CNI (Segundo et al., 2006; Noris et al., 2007). Thus, experimental and clinical evidence suggests that rapamycin is tolerogenic by favoring Tregs, supporting its use as an adjunct therapy with Tregs.
A new generation of biologics, such as anti-CD25 mAb, CTLA-4Ig, anti-LFA-1 mAb, LFA-3Ig, and anti-CD20 mAb, that specifically target the immune system are being tested in transplant patients (Vincenti and Kirk, 2008; Sanoudj et al., 2010; Weclawiak et al., 2010) and their effects on Tregs are just beginning to be characterized. Anti-CD25 mAb was developed with the intention to target recently activated effector T cells in transplant patients prior to the realization of constitutive expression of this marker on Tregs. Anti-CD25 mAb are widely used to deplete Tregs to boost immune response in mouse models. Its use in transplant patients also leads to transient loss of Tregs in circulation (Bluestone et al., 2008; Tosó et al., 2009), but the therapy is surprisingly innocuous, and even beneficial, for transplant recipients (Bumgardner et al., 2001; Kandus et al., 2010). This may be due to the more efficient depletion of CD25+Foxp3− effector T cells and only transient and partial depletion of Tregs (Bluestone et al., 2008). While more in-depth characterization of the effects of anti-CD25 mAb on Tregs and Tconv cells in transplant patients is needed to fully appreciate its action in transplant patients, it may be best to avoid the use anti-CD25 mAb together with Treg therapy.

Thymic development and peripheral homeostasis of Tregs depends on CD28; thus CD28-deficient mice have significant defects in Tregs and develop a wide range of autoimmune diseases on an autoimmune-prone background (Tang et al., 2003). Similarly, blocking CD28 signaling using CTLA-4Ig or anti-CD80 and anti-CD86 mAb leads to reduction in Tregs by blocking their renewal and enhancing their apoptosis. CTLA-4Ig treatment in transplant patients was not associated with a loss of Tregs, likely because the dose administered was not sufficient to saturate all CD80 and CD86-binding sites in vivo (Bluestone et al., 2008). In mouse models, partial blocking of CD80 and CD86 can effectively prevent Tconv cell activation without impairing Treg homeostasis (Tang et al., 2004a), thus non-saturating dose of CTLA-4Ig may synergize with Treg therapy. A recent report on the use of anti-LFA-1 mAb in islet transplant patients showed dramatic increase in circulating Tregs in all patients, suggesting that LFA-1 blockade may also favor Tregs (Posselt et al., 2010). As more new therapies are being tested in transplant patients, careful immune monitoring of effects of the new agents on Tconv cell and Treg alloimmune responses will be instrumental in assessing their tolerogenic potential and their potential utility as adjunct therapy with Tregs.

In summary, preclinical investigations have clearly established the efficacy and feasibility of Treg therapy for controlling allograft rejection and inducing transplant tolerance in animal models. Alloreactive Tregs are present in a normal Treg repertoire and new specificities can be acquired through infectious tolerance under tolerogenic conditions. The low frequency of Tregs can be overcome using short-term ex vivo expansion. Infusion of Tregs, when combined with proper pre-conditioning and/or adjunct immunosuppression, can confer indefinite graft survival in various transplant models. Although more work is needed to determine optimal adjunct therapy and the stability of the infused Tregs in the transplant setting, there is now ample evidence to support the translation of this approach to the clinical arena.

**Translating Treg therapy to the clinic**

**Early human experience in GvHD**

As noted above, Treg therapy in animal models of tissue transplantation has been efficacious. However, the therapeutic ratio of Treg to Tconv cells needed for efficacy was usually equal to or greater than one to one, even as high as ten to one. The need for such non-physiologically high percentages of Tregs in these settings is not clear, but may be in part related to the high frequency of alloreactive Tconv cells, contamination of Treg populations with effector T cells, as well as the ‘danger’ signals that are elicited by transplantation, which have been shown to antagonize Treg function and potentially generate Treg-resistant effector T cell subsets. Although such high ratios of Tregs to Tconv cells are impossible to achieve in lymphoreplete recipients, these observations are not without clinical relevance. Bone marrow or hematopoietic stem cell transplantation is a common treatment for patients with congenital immunodeficiency or those with hematological malignancies whose own immune system is destroyed along with the cancer by chemotherapy and/or irradiation. In both settings, T cells from the donor are often infused along with stem cells to improve engraftment of the transplanted stem cells. In the malignancy setting, the donor T cells also help to eliminate residual cancer cells because the host-derived cancer is allogeneic to the donor-derived T cells, a phenomenon referred to as graft-versus-leukemia (GvL) effect. However, donor T cells can also recognize and damage healthy host tissues leading to GvHD, which can be fatal in the most severe cases. In mouse models of GvHD, co-transfer of donor Tregs dramatically suppresses GvHD without impairing the beneficial engraftment and GvL effects and responses against unrelated alloantigens (Cohen et al., 2002; Taylor et al., 2002; Edinger et al., 2003; Joffre et al., 2004).

Within 8 years of the first demonstration of the efficacy of Tregs in suppressing GvHD in mouse models, three trials of Treg therapy for GvHD in patients have been reported (Trzonkowski et al., 2009; Brunstein et al., 2010; Di Ianni et al., 2011). The first-in-man trial by Trzonkowski et al. (2009) involved two patients. The first patient had chronic GvHD 2 years after transplantation. After receiving $0.1 \times 10^8/kg$ fluorescence-activated cell sorting (FACS) purified ex vivo expanded Tregs from the donor, the symptoms subsided and the patient was successfully withdrawn from immunosuppression. The second patient had acute disease that progressed despite three infusions with an accumulative dose of $3 \times 10^8/kg$ expanded donor Tregs. A larger scale phase I trial by Brunstein et al. (2010) has recently been concluded. Twenty-three patients with advanced hematologic malignancy were enrolled and treated with two units of umbilical cord blood as source of stem cells and effector T cells. Tregs were isolated using anti-CD25 immunomagnetic bead selection from third-party cord blood samples that had 4–6 HLA match with the recipient. Up to $6 \times 10^6/kg$ Tregs, expanded ex vivo using anti-CD3 and anti-CD28 conjugated beads, were infused. The infused Tregs were detectable in circulation for up to 7 days. During the 1-year period after Treg infusion, the investigators observed no dose-limiting toxicities or increase in adverse events when compared with historical controls. Incidences of severe acute GvHD were significantly reduced in patients who
received Treg therapy. The third trial enrolled 28 patients with high-risk hematological malignancies (Di Ianni et al., 2011). Patients received anti-CD25 immunomagnetic bead-enriched donor Tregs without ex vivo expansion 4 days before receiving one haplo-mismatched hematopoietic stem cell and Tconv cell transplants from the same donors. The majority of the patients received $2 \times 10^6$/$kg Tregs with $1 \times 10^6$/$kg Tconv cells. No adjunct immunosuppression was given after transplant. Patients demonstrated accelerated immune reconstitution, reduced CMV reactivation, and a lower incidence of tumor relapse and GvHD when compared with historical controls. These encouraging early experiences in patients support further investigation of the efficacy of Treg therapy in controlling GvHD and applying Treg therapy in other disease settings.

Treg therapy in other indications: need for Treg expansion

One of the obstacles in developing Treg therapy for most transplant and autoimmune settings is their low abundance, especially antigen-specific Tregs. Technical advances in ex vivo Treg expansion in the past decade have made it feasible to consider Treg therapy (Levings et al., 2001; Cohen et al., 2002; Taylor et al., 2002; Yamazaki et al., 2003; Hoffmann et al., 2004; Tang et al., 2004b; Earle et al., 2005). In general, Tconv cells outgrow Tregs in vitro; therefore, high purity of Tregs is needed as a starting population. CD25 does not identify a distinct Treg population in human CD4+ T cells. The addition of CD127 as a marker significantly improves the purity of Tregs and their efficacy in suppressing allograft vasculopathy in humanized mouse models (Liu et al., 2006; Nadig et al., 2010). Isolating Tregs based on three surface markers is cumbersome using immunomagnetic bead selection, a method preferred due to its low instrument cost and good manufacturing practice-compliant status. FACS-based isolation can provide high-yield CD4+CD25+CD127lo Tregs for ex vivo expansion of highly pure Tregs (Putnam et al., 2009) and this protocol has been approved by FDA for a phase I safety trial in type 1 diabetic patients (NCT01210664).

Despite these technical advances, large-scale Treg manufacturing remains challenging because of limited expansion in short-term cultures and outgrowth of Tconv cells. Two approaches have been proposed to overcome these challenges. First, since CD4+ Tconv cells can be readily expanded, protocols have been developed using Tconv cells as a starting population, with the hope of converting them to Tregs during the expansion using skewing mix containing TGF-β and/or retinoic acid, by using tolerogenic antigen presenting cells, or by lentiviral transduction of Foxp3 (Hori et al., 2003). However, as discussed above, these induced adaptive Tregs are not stably committed and may lose their suppressive activity or even take on an effector phenotype. Additional inclusion of chemicals that promote chromatin demethylation and protein acetylation may help adaptive Tregs to commit to the Treg lineage; however, their stability in vivo remains to be demonstrated. Second, large-scale expansion of Tregs can be achieved with repetitive stimulations (Cohen et al., 2002; Hoffmann et al., 2004; Hippen et al., 2011); however, the percentage of Foxp3+ cells decreases with more than two rounds of weekly stimulations, correlating with increased methylation of the Foxp3 promoter locus (Hoffmann et al., 2009). Interestingly, resting Tregs identified by CD45RA expression are more stable in culture. This result suggests that prolonged in vitro culture destabilizes Tregs, and restricting the starting population to those that express CD45RA or limiting in vitro stimulation may help to retain Treg phenotype. Related to this issue is the observation that inclusion of rapamycin helps to preserve Treg phenotype and function in long-term in vitro cultures (Hippen et al., 2011). This effect may be due to the differential sensitivity of Tregs and Tconv cells to the anti-proliferative function of rapamycin (Battaglia et al., 2005). Alternatively, but not mutually exclusively, rapamycin may promote the conversion of Tconv cells to Tregs (Delgoffe et al., 2009). It is important to determine the stability of the Tregs expanded with rapamycin, especially considering that the concentration used in the expansion cultures (~100 μg/L) is 10 times higher than in vivo trough levels in patients (8–12 μg/L). A recent report demonstrates that Tregs expanded short term in rapamycin do not make more effector cytokines and retained the Treg phenotype when transferred into mice without rapamycin treatment (Tresoldi et al., 2011). Such analyses and assessment of Foxp3 promoter demethylation status are important and should be performed on long-term rapamycin cultured Tregs. Overall, clinical-grade Treg expansion protocols should maximally balance yield, purity, and stability of Tregs.

Since donor-specific Tregs are more effective in controlling graft rejection in preclinical models than non-specific Tregs, and less likely to confer non-specific global immunosuppression, the use of donor-specific Tregs is preferred. It is feasible to expand Tregs with direct alloantigen specificity owing to their high precursor frequency. However, Tregs with indirect alloantigen specificity are more challenging to expand in short-term cultures (Gupta et al., 2011). A protocol that can reliably produce large numbers of clinical grade, highly pure, and stable donor-specific Tregs using short-term cultures is much needed to enable clinical testing of their safety and efficacy in transplant patients. In this regard, we have developed a protocol that selectively expands donor-specific Tregs up to 1000 folds in <20 days. Contrary to the dogma that dendritic cells are most efficient at expanding T cells, we found that CD40 ligand-stimulated human B cells are extremely potent at inducing proliferation of Tregs. Tregs expanded with the stimulated B cells are virtually all donor-specific, exhibit potent donor-specific suppressive activities, and have demethylated TSDR (our unpublished data). This protocol is currently being adapted to a fully good manufacturing practice-compliant platform for clinical applications.

As an alternative to selective expansion of antigen-specific Tregs, forced expression of genes for TCR with known specificity during expansion has been used experimentally to confer desired specificity to polyclonally expanded Tregs (Brusko et al., 2010). Tsang et al. (2008) combined selective expansion of direct alloreactive Tregs and transferring TCR with indirect specificity and find that the engineered dual-specificity Tregs have improved ability to protect grafts when compared with Tregs with only direct donor specificity. In addition to TCRs, genes for traceable markers and drug-inducible suicidal enzymes can also be introduced by genetic modification of Tregs to allow monitoring of the infused Tregs and their elimination when needed. However, significant complexity as well as
safety concern is added by genetic modification of cells for human therapy.

Concluding remarks

Compelling evidence from animal models demonstrates a key role for Tregs in transplantation tolerance and efficacy of Treg therapy in preventing rejection and inducing tolerance. Dares of preclinical investigations provide a strong framework for testing Treg therapy in humans. Recent results from the bone marrow transplant arena demonstrate promising safety and possibly some efficacy in human patients and provide further support for expanding Treg therapy testing in other settings such as solid organ transplantations. Since Treg therapy in humans is still a nascent field, initial clinical trials for administering Tregs to transplant recipients should involve a small number of patients aiming at evaluating the safety of increasing doses of Tregs. We believe that an ideal trial should have three interconnected components. First, a clinical protocol should be based on a ‘Treg-supportive’ immunosuppressive regimen, which would provide an element of safety against rejection while maximizing the potential efficacy of exogenously administered Tregs. Second, it is essential to have a robust Treg manufacturing protocol with release criteria set with the most current understanding of Treg biology. Third, a comprehensive immune monitoring plan of patients should be an integral part of a Treg therapy trial to gain mechanistic insight on Treg function in patients. Carefully planned and expertly executed trials will not only pave the way for Treg therapy for inducing transplantation tolerance in patients with end-stage organ diseases, but also extend our knowledge of Treg biology in humans.

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References


Regulatory T cell therapy in transplantation


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